Supporting Information

Lin et al. 10.1073/pnas.0803060105

SI Materials and Methods

Neuronal Experiments. Hippocampal neurons were dissected from postnatal day 0 or 1 rat pups and cultured on poly-D-lysine-coated plastic plates in Neurobasal medium supplemented with B27 and glutamine. For immunoblotting experiments, neurons were transfected before plating using the Amaxa Nucleofector protocol. For other experiments, neurons were transfected at 7–10 days *in vitro* (DIV) by the calcium phosphate method.

For time lapse imaging, we chose 14–21 DIV neurons with pyramidal morphology expressing PSD-95-GFP-TimeSTAMPa-HA with dim GFP fluorescence. These were imaged for GFP by epifluorescence on a Zeiss Axiovert 200M with a temperature control chamber at 37°C and a 100× oil objective in HBSS supplemented with B27 and 10 μM BILN-2061. For each position and time point, a stack of 20 images spaced 0.5 μm apart through the neurons was acquired. After fixation with 4% paraformaldehyde for 10 min, neurons were stained for HA and synapsin by standard protocols and then imaged again for GFP, HA, and synapsin. Image stacks were cropped to remove sections lost to focal drift and flattened into single maximum projection images for analysis.

For synaptic density quantification in neurons transfected with various fusions of PSD-95-GFP to NS3, maximum intensity projections of stacks of 20 images spaced 0.5 μm apart of 14 DIV neurons at 7 days posttransfection (DPT) were acquired of GFP fluorescence and synapsin immunofluorescence in a blinded manner. In ImageJ software, a 60-μm-long segment of the primary dendrite beginning 30 μm from the cell body was traced in the GFP channel and then dilated by 1 μm and used as a positive mask for the synapsin channel. Synapsin staining within the mask was isolated by using the automatic threshold function. Synaptic density was defined as the area covered by synapsin staining, as calculated by using the analyze particles function, divided by the mask area.

For EosFP photoconversion experiments, because photoconversion is most effective at pH <7, neurons expressing proteins fused to tdEosFP were moved into HBSS (pH 6.9) supplemented with B27 as a source of antioxidants. Focal photoconversion was performed in a temperature control chamber at 37°C on an inverted microscope using illumination from a xenon arc lamp passing through a 420/20-nm bandpass filter, a stopped-down diaphragm, and a 100× oil objective. Under these conditions, red fluorescence increases to 4-fold over beginning levels by 1.5 min of illumination, remains constant over the next 1.5 min, then falls, presumably because of photobleaching. After undergoing rapid and variable photoactivation, green fluorescence drops to 0.5× of maximal values by 1.5 min, then to 0.35× by 3 min. Further illumination was associated with blebbing of illuminated neurites. To minimize phototoxicity and maximize red fluorescence marking locally converted protein, we therefore performed photoconversion for 3 min. Global photoconversion was performed on a solar simulator with a xenon arc lamp passing through a 420/40 bandpass filter for 40 min, resulting in a 9-fold increase in red fluorescence and a final green fluorescence 10% of beginning values. After photoconversion, neurons were returned to conditioned Neurobasal medium with B27 and maintained at 37°C and 5% CO_2 and imaged in HBSS at various times afterward.

Fly Experiments. Homozyous transformed lines were established from single progeny of embryos injected with pUAST-HSV-TimeSTAMPt-HA-dCaMKII or pUAST-HSV-TimeSTAMPa-HA-dCaMKII. Homozygotes showed no behavioral or fertility phenotypes, and no loss of P-elements or transposition to other chromosomes were observed in balanced lines. Homozygous adults had smooth eyes, and brains were normal in size. Male homozygotes with third-chromosome insertions were crossed to elav-GAL4;UAS-tubulin-GFP;+ females, and the male elav-GAL4/Y;UAS-tubulin-GFP/+;HSV-TimeSTAMP-HA-dCaMKII/+ progeny were used for experiments between 1 and 2 days after eclosion.

For experiments involving HSV-TimeSTAMPt-HA-dCaMKII, flies were anesthetized by carbon dioxide, then a hole was punctured in the medial ocellus region using a glass micropipette with a 10-μm bore, and a 25-nl drop of 2 mM BILN-2061 in 20% DMSO 5% Cremophor EL in HBSS was placed over the region. The drug solution was observed to be absorbed within 2 min, then flies were returned to food vials with a wetted plug and allowed to recover. After recovery, flies were observed to feed, fly, and engage in courtship behavior. For HSV-TimeSTAMPa-HA-dCaMKII, flies were starved for 12 h with only water and then placed in a vial with an emulsion of 30% (wt/vol) yeast, 30% (vol/vol) glycerol, 30% (vol/vol) water, 5% green food coloring, 5% dimethylformamide, and 500 μM BILN-2061. Most flies were observed to ingest the food coloring within 15 min. For analysis, flies were immersed and rapidly decapitated in fixative (HBSS with 4% paraformaldehyde and 0.2% Triton X-100), and brains were dissected and incubated in fixative for a total of 40 min at room temperature. Brains were processed for immunocytochemistry by standard methods and imaged on a Zeiss LSM510 or LSM5Live confocal microscope.

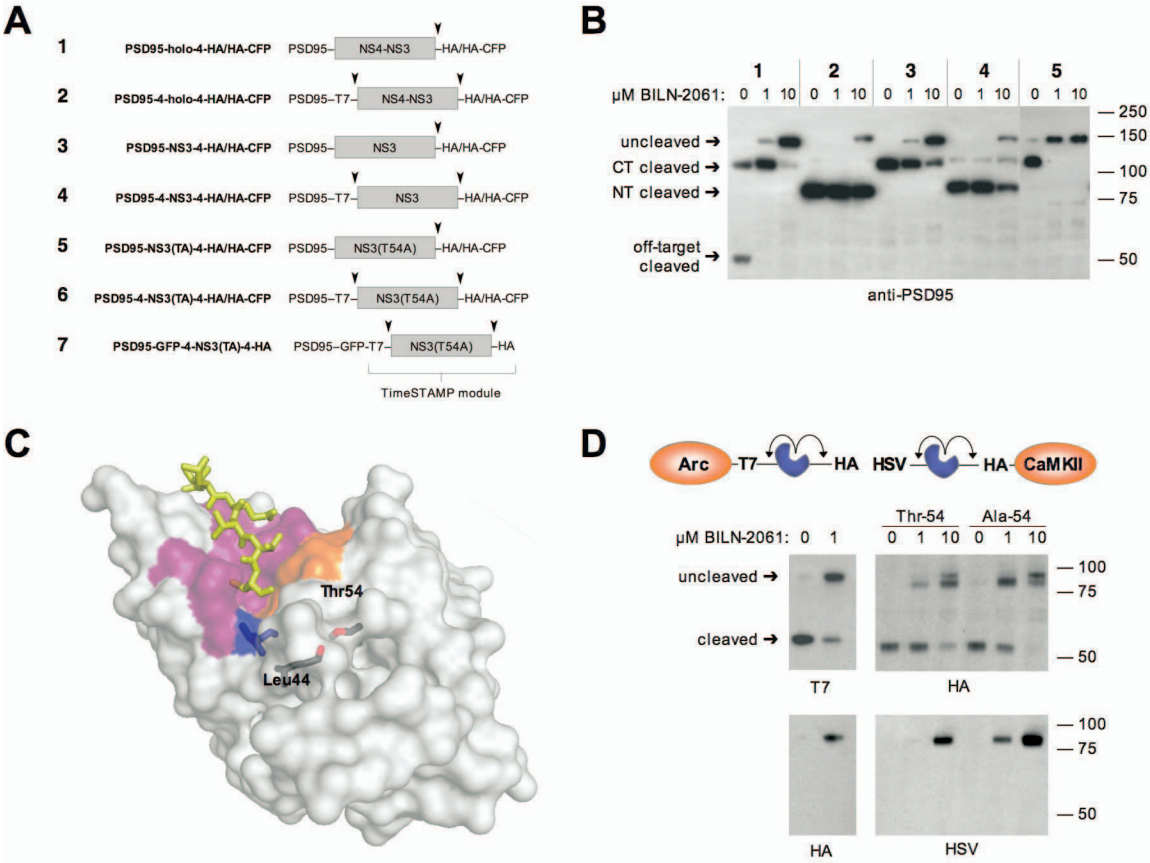

Fig. S1. Optimization of TimeSTAMP. (*A*) Organization of fusion proteins used in *B* and Fig. S2C. (*B*) Cyan fluorescent protein-containing constructs 1–5 from *A* were expressed in HEK293 cells in 0, 1 μM, or 10 μM BILN-2061, and cleavage of the constructs was assayed by immunoblotting. (*C*) The T54A reduced activity mutation is unlikely to affect substrate or BILN-2061 binding. Substrate-bound NS3 was rendered based on coordinates from Protein Data Base ID code 1CU1. A P1 to P6 substrate is shown as yellow sticks. The BILN-2061 contact surface, the catalytic triad, and the oxyanion hole are purple, orange, and blue, respectively. The backbone atoms of the oxyanion hole residues are shown as blue sticks. The side chain and backbone carbonyl of Leu-44 and the side chain of Thr-54 are shown as sticks with carbon atoms in black, oxygen in red, and hydrogen in white. (*D*) The TimeSTAMP module functions at either the N terminus or C terminus. HEK293 cells expressing Arc-TimeSTAMPa-HA at 37°C (*Left*) or HSV-TimeSTAMPt-HA-dCaMKII or HSV-TimeSTAMPa-HA-dCaMKII at 25°C (*Right*) in the continual absence or presence of BILN-2061 were analyzed by immunoblotting.

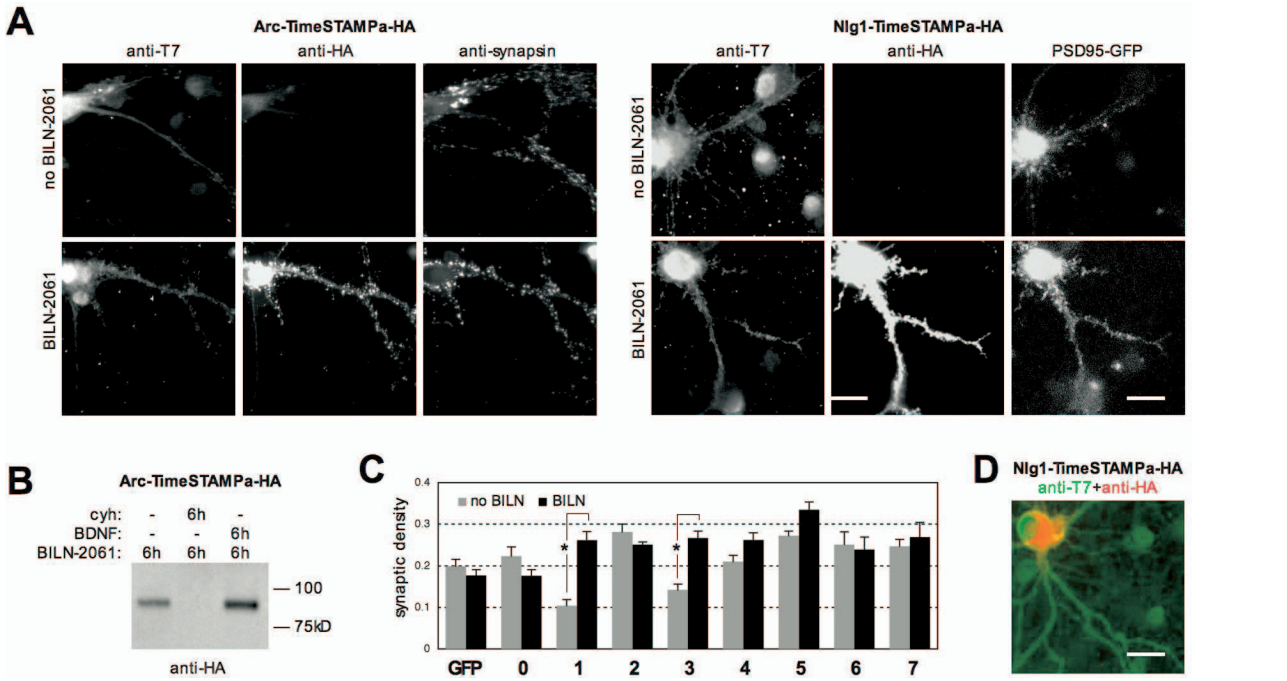

Fig. S2. TimeSTAMP is generalizable and functions in neurons without toxicity. (*A*) Neurons were transfected at 9 DIV with fusions of Arc (*Left*) or Neuroligin1 (Nlg1; *Right*) to TimeSTAMPa-HA and grown in the absence or presence of BILN-2061 for 3 days. To mark synapses, Nlg1-TimeSTAMPa-HA-expressing neurons were cotransfected with PSD-95-GFP, and Arc-TimeSTAMPt-HA-expressing neurons were stained for synapsin. T7 is a constitutive tag located N-terminal to the left cleavage site, and HA is drug-dependent. Anti-T7 cross-reactivity to the nucleus has been previously observed in various cell types. (*B*) TimeSTAMP detects stimulus-dependent new protein synthesis in neurons. Neurons were transfected by Amaxa nucleofection at 0 DIV with Arc-TimeSTAMPa-HA and analyzed at 7 DIV. HA-tagged Arc appearing after incubation in 10 μM BILN for 6 h was blocked with simultaneous cycloheximide (cyh; 50 μg/ml) treatment and increased with simultaneous BDNF stimulation. (*C*) Quantification of synaptic density in cells transfected with the PSD-95 fusions shown in *A*. Each condition contained 5 neurons scored blinded. Differences were significant by ANOVA ($P = 0.0057$). Only permanent fusions of wild-type NS3 show significantly lower synaptic density in the absence of inhibitor ($P < 0.05$ on pairwise *t* tests, asterisks). Data represent mean $\pm$ SEM. (*D*) Nlg1-TimeSTAMPa-HA reveals distribution of newly synthesized Nlg1 after 6 h. (Scale bars, 20 μm.)

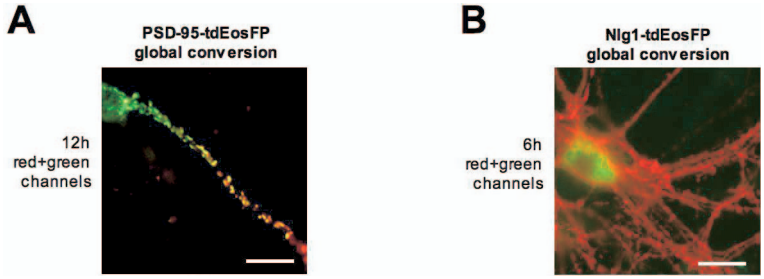

Fig. S3. Validation of protein movements using the photoconvertible protein tdEosFP. (*A*) After PSD-95-tdEosFP in 12 DIV neurons was photoconverted globally to red, new green protein was observed in a gradient from the cell body 12 h later, confirming results obtained by TimeSTAMP. (*B*) Similarly, 6 h after photoconversion, new Nlg1-tdEosFP protein was observed in the soma in a perinuclear distribution consistent with movement through the secretory pathway. (Scale bars, 20 μm.)

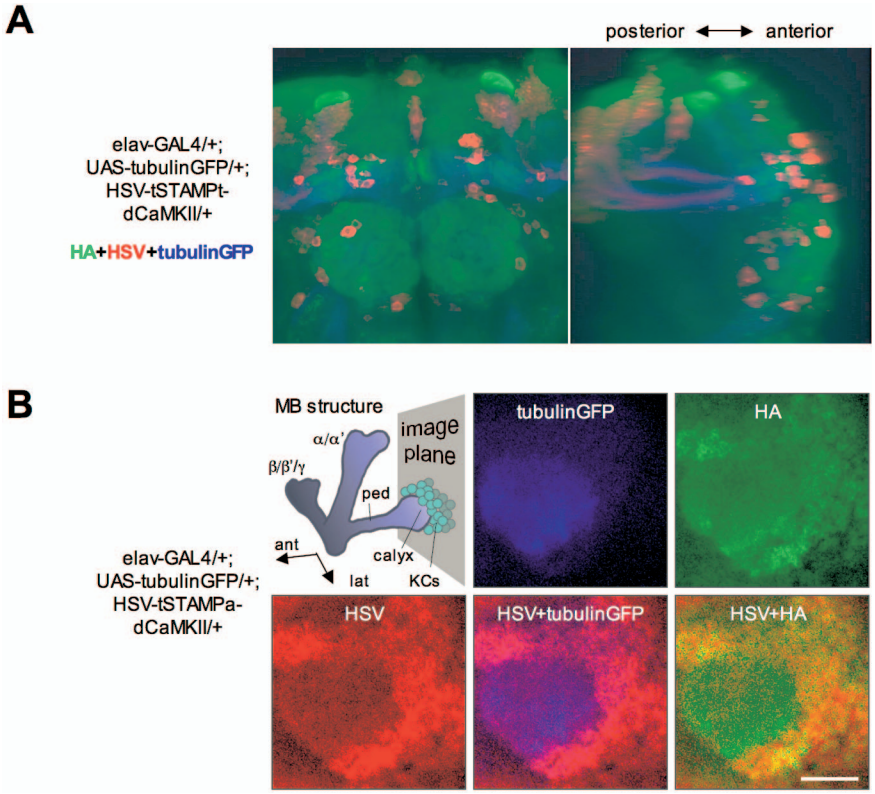

Fig. S4. TimeSTAMP reveals tissue and subcellular locations of newly synthesized dCaMKII. (*A*) A three-dimensional map of 6-h fractional new dCaMKII was constructed in flies expressing HSV-TimeSTAMPt-HA-dCAMKII. Anti-HSV labels new dCaMKII, and anti-HA labels total dCaMKII. Front and side projections of confocal sections spaced 2 μm apart are shown. (*B*) HA staining of flies expressing HSV-TimeSTAMPa-HA-dCaMKII shows total dCaMKII in Kenyon cell bodies and within the calyx, which is enriched in tubulin-GFP. HSV staining reveals recently synthesized dCaMKII is enriched in the cell bodies. (*Upper Left*) Diagram of the left mushroom bodies. ped, peduncle; KCs, Kenyon cell bodies; ant, anterior; lat, lateral. (Scale bar, 10 μm.)